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Cold injury is often associated with irreversible cell damage. To date, however, the pathophysiology of cold injury is not known. The present study examines the mechanism of such injury. New Zealand white rabbits were anesthetized with ketamine and xylazine and then ventilated using a Harvard ventilator. The femoral artery and vein were exposed. A flowprobe was placed around the femoral artery which, in turn, was connected to a six-channel recorder. A continuous display of EKG was obtained on lead II by connecting the limb leads to the same recorder. A thermocouple probe was inserted inside the leg to be cooled. After baseline measurements, one leg was cooled with a freezing mixture up to 0°C, which was followed by rewarming. The other leg served as control. During the experiment, blood samples were withdrawn from the femoral artery and vein for the subsequent analysis of creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA). At the end, salicylate was injected through the femoral vein to trap any hydroxyl radical (OH·) formed. Rabbits were immediately sacrificed, and biopsies were withdrawn

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19. Abstract (continued)

and frozen at liquid N_2 temperature to analyze $OH^{\cdot}$ and high energy phosphate compounds. Our results indicated that local blood flow in cold-exposed leg was reduced significantly, suggesting that cold injury was associated with ischemic insult. The flow was reduced from 2.5 ml/min (control) to 0.8 ml/min at 0°C and then increased up to 2.2 ml/min upon rewarming. CK and LDH were increased after cold exposure and were increased further during rewarming, suggesting injury to the cells. MDA formation followed a similar pattern, indicating formation of lipid peroxidation. $OH^{\cdot}$ generated after cooling increased significantly (2.5 times) upon rewarming. These results indicate that rewarming is associated with an episode of ischemia/reperfusion with simultaneous generation of free radicals, which at least in part may be responsible for the cellular injury.

In essence, during the second year we will continue to explore the mechanism of NFCI, with particular reference to membrane phospholipid breakdown and NEFA accumulation. In addition, we will explore various therapies to prevent NFCI. These therapies will include various free radical scavengers and phospholipase and thromboxane synthase inhibitors.

PUBLICATIONS AND REPORTS (Year 1)

1. We are presenting a portion of our data at the Annual Meeting of Cryobiology (Cryo '89) in Charleston, South Carolina, June 11-16, 1989. This talk has been scheduled for June 15th, 8:30 a.m.

2. An abstract has been submitted for the 36th Annual Meeting of the American College of Angiology (copy enclosed). I have nominated Dr. S. Samanta, who is working on this project, for the Young Investigator Award. For this reason, a manuscript (extended abstract) has been submitted by Dr. Samanta (copy enclosed). If Dr. Samanta is selected for the award, the manuscript will automatically be published; otherwise, I will submit it to *Cryobiology*.

3. An Annual Report will be distributed to the ONR distribution list at the anniversary date, as required.

TRAINING ACTIVITIES

Two Fellows, one full-time and one part-time, are working on this project. The demographic data regarding these Fellows are:

Women or minorities: 2

NOTE TO SCIENTIFIC OFFICER

The progress of this research has been outstanding. However, we have had some difficulty in measuring free radicals directly because of the unavailability of an EPR spectrometer. This was mentioned in my original application. Is there any possibility that funding for an EPR will be reconsidered?

ABSTRACT

The pathophysiology of cold injury was examined by cooling a hind leg of an anesthetized New Zealand white rabbit. A flow probe and a thermocouple were placed in the leg to be cooled to monitor the blood flow and tissue temperature. After baseline measurements, the leg was cooled with a freezing mixture up to O°C, which was followed by rewarming. The other leg served as control. In the experimental group, liposome-bound superoxide dismutase and catalase were infused through the femoral vein 15 minutes prior to putting the freezing mixture on the leg. Salicylic acid was injected through the femoral vein at the end of some experiments to assay hydroxy radical ($OH^{\cdot}$). Our results demonstrated reduction of local blood flow in cold-exposed leg, indicating development of ischemia. Creatine kinase and lactate dehydrogenase were increased during rewarming in conjunction with hydroxyl radical formation, phospholipid breakdown, and lipid peroxidation. Treatment with superoxide dismutase and catalase reduced $OH^{\cdot}$ formation, prevented phospholipid degradation, and decreased creatine kinase, lactate dehydrogenase, and malonaldehyde formation. These results indicate that rewarming of cooled tissue is associated with rewarming injury similar to reperfusion injury, and that oxygen-derived free radicals play a significant role in the pathophysiology of such injury.

INTRODUCTION

Non-freezing cold injury (NFCI) represents a potential threat to infantry and marine operations carried out in inclement weather conditions. The term NFCI is used to identify the syndrome that results from damage to tissues that have been cooled, usually for prolonged periods, at temperatures between about 15°C and their freezing point -0.5°C. NFCI reduces the man's mobility at the time, but it may, through cold sensitization, compromise his ability to fight in similar conditions in the future.

Very little work has been done to understand the pathophysiology of NFCI. During NFCI, intracellular water moves to the interstitial space, and the cells become hyperosmolar and then freeze. Vascular insufficiency occurs in the rewarming phase primarily because of arteriolar spasm, presumably due to thrombosis, sludging of red cells in small vessels and shunting of blood past the capillaries by arteriovenous communications.

Another major problem is that during NFCI, the hemoglobin dissociation curve is shifted to the left, thereby preventing release of O_2 to the tissue. This combination of poor perfusion of capillaries and decreased ability to release O_2, induces severe ischemic insult to the affected tissues despite the fact that hypothermia reduces the rate of cellular metabolism significantly. Almost any part of the body, including nerve fibers, muscle, skin, soft tissue, and even bone can be injured. Blood vessel injury is severe, and is manifested by severe spasm of small arteries and arterioles, and increases permeability or leakiness of the capillary bed on rewarming of the frozen part. Poor perfusion, anoxia and flow through cold-injured vessels cause aggregation of red cells, platelets, and polymrphonuclear leukocytes (PMNS), leading to thrombosis with tissue infarction (gangrene).

Once NFCI occurs, the only remedy remains to rewarm the affected areas. Therefore, all the attempts for improved therapy have been directed at improving vascular flow and tissue perfusion after slow or rapid warming has occurred[1,2].

Although this phenomenon has never been explored in conjunction with NFCI, it is quite possible that NFCI-induced ischemic tissue is subjected to the so-called "reperfusion injury" during the rewarming phase. Unless the tissue has already gone through fourth degree of NFCI leading to complete necrosis

on rewarming, the part of the affected organ becomes hyperemic and edematous as a result of the re-flow phenomenon. Current research has pointed to "reperfusion injury", clearly distinctive from "ischemic injury" associated with the revascularization phase[3,4]. A clear role of free radicals has been indicated in the pathogenesis of reperfusion injury[5-7]. This study examines whether a similar phenomenon occurs during rewarming of cooled tissues, and if free radicals have any role in the pathophysiology of NFCI.

MATERIALS AND METHODS

Animal Preparation

New Zealand white rabbits of about 2.5 kg body weight were anesthetized with xylazine (5 mg/kg) and ketamine (30 mg/kg) and were maintained under anesthesia during the entire experiment. An electric clipper was used to remove hair from the hind limb as well as from the front of the neck area. A tracheostomy was performed, and the rabbits were ventilated by a Harvard ventilator (15 ml volume and 50 strokes/min). Femoral arteries and veins of both sides were exposed and dissected free of tissue. A flow probe (Transonic, Ithaca, NY) was placed around the femoral artery, which in turn was connected to a six-channel simultrace chart recorder (Honeywell Inc., Pleasantville, NY). The femoral vein of the same side was cannulated with an I.V. placement catheter for withdrawal of blood samples. Another such catheter was placed in the femoral artery of the other limb. A continuous display of electrocardiogram (EKG) was obtained on Lead II by connecting the limb leads to the same recorder. The baseline values were established by measuring EKG and blood flow, as well as by estimating creatine kinase (CK), lactate dehydrogenase (LDH), and malonaldehyde (MDA) from the arterial samples. The experimental animals were divided into three groups. One group served as control. The other group was treated with free radical scavengers, SOD plus catalase bound to liposome. The third group was treated with lipose only. The interventions were administered through the femoral vein. The leg was then cooled down to 0°C with a freezing mixture containing ice and salt. Continuous monitoring of the interstitial temperature was achieved by inserting a thermocouple probe (Omega Engineering, Inc., Stamford, CT) into the limb. Tissue temperature was maintained for 20 min at 0°C. The ice was

then removed, and the limb was allowed to rewarm to room temperature. During the experiment, blood samples were withdrawn at regular intervals of time for the subsequent assay of CK, LDH, and MDA formation. Blood flow was also continuously monitored. At the end of the experiment, salicylate (2 mM) was injected through the femoral vein to trap free radical. The rabbits were immediately sacrificed by an overdose of sodium pentobarbital. Tissue biopsies were withdrawn for assay of $OH^{\cdot}$.

Assay for $OH^{\cdot}$

The method used to trap and quantitate $OH^{\cdot}$ was similar to that described by Grootveld and Halliwel[8]. The tissue was homogenized under liquid N_2. The ground tissue was suspended in a buffer containing a mixture of sodium citrate (0.05 M) and sodium acetate (0.03 M) (pH 4.5); 50 μl of 70% perchloric acid was then added to the mixture. The resultant mixture was degassed and filtered through a Rainin Nylon-66 membrane filter (0.45 μM). The sample (20 μl) was injected onto an Altex Ultrasphere 3 μODs (75 x 4.6 mm) equipped with a Water Associates HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. The hydroxylated products of salicylic acid were eluted with buffer (degassed and filtered) containing 0.03 M sodium acetate and 0.05 M sodium citrate (pH 4.5) at a flow rate of 0.8 ml/min. The detector potential was maintained at 0.6 V, employing Ag/AgCl reference electrode.

Measurement of Lipid Peroxidation

Malonaldehyde was measured as an index for lipid peroxidation. Plasma (0.5 ml) was added to 0.5 ml ice-cold perchloric acid (15%) and then treated with 0.75% thiobarbituric acid (TBA) as described previously[9]. Samples were boiled for 20 min and centrifuged to remove the pellet. The color of the supernatant was read at 535 nm. The concentration of MDA (nmol/ml) was calculated by using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

Assay for CK and LDH

CK and LDH were assayed in plasma samples obtained from the femoral artery, using an assay kit obtained from Sigma Chemical Company (St. Louis, MO) as described elsewhere[10].

Measurements of Phospholipids

At the end of each experiment, tissue biopsies were frozen in liquid nitrogen. Lipids were extracted with chloroform-methanol mixture by the method of Folch *et al.* Phospholipids, except for lysophosphatidylcholine (LPC), were separated on silica K6 plates (Whatman, Clifton, NJ) using a mixture of chloroform-methanol-petroleum ether-acetic acid-boric acid (40:20:30:10:1.8, vol/vol/vol/vol/wt) as a solvent system[11]. LPC was separated on silica gel H plates (Analtech, Newark, DE) using a mixture of chloroform-methanol-aetic acid-water (75:25:3:4, vol/vol/vol/vol) as a solvent system. Neutral lipids were separated on silica GF plates (Analtech) using a mixture of hexane-diethyl ether-acetic acid (70:30:1, vol/vol/vol) as a developing solvent. The lipids on the silica gel plates were identified by cochromatography with authentic lipid standards after brief exposure with iodine vapor, scraped off, and quantitated by the method of Bartlet[12].

RESULTS

Monitoring of Heart by EKG

The electrocardiographic pattern showed regularity in rate and rhythm of heart during the entire period of cooling the leg from 28°C to O°C (Fig. 1). Heart rate was always maintained in a steady condition even during rewarming after keeping the limb at O°C for 20 min. There was no difference in heart rates among the control, liposome, and SOD-catalase groups.

Blood Flow During Cooling and Rewarming

Hemodynamic changes in the femoral artery were continuously monitored with the flow probe. The flow pattern was also recorded during the experiment (Fig. 2). Under normal conditions, the flow rate was about 3.5 ml/min. During cooling of the limb, the flow rate continuously dropped. By the time the interstitial temperature became O°C, it decreased to about 28% of the normal value to 1 ml/min (Fig. 3). No difference was found in any of our results between the control and liposome groups, and therefore, we have used liposome group as control in all of our results. A steady low flow rate was maintained for the rest of the cooling period. When rewarming was initiated by removing the freezing

mixture from the leg, the flow rate gradually increased even when the interstitial temperature remained at 0°C. The flow rate continued to rise, and at the end of the rewarming the flow rate was compietely restored. Slightly higher flow was observe during rewarming in the SOD plus catalase group, but the difference was not statistically significant.

Release of LDH and CK

The release of arterial plasma LDH is plotted against temperature during cooling and rewarming of the rabbit leg (Fig. 4). There was no change in plasma LDH levels during cooling from 30°C to 15°C, but below that temperature there was a slight increase in LDH release. A remarkable increase in plasma LDH (about 2-fold) was noted at the end of rewarming, suggesting that tissue damage occurred mostly during the rewarming period. SOD plus catalase prevented this increase in LDH release significantly. CK, another marker for tissue necrosis, followed a similar pattern (Fig. 2). After cooling, a slight increase in CK activity was noticed, but the differences were not statistically significant. During rewarming, however, CK increased dramatically. At the end of reperfusion, these values were 2.5-fold higher compared to the baseline levels. Once again, the enhanced CK release was significantly blocked by SOD plus catalase.

Estimation of Lipid Peroxidation

Malonaldehyde formation, a presumptive marker for lipid peroxidation, remained unchanged during cooling (Fig. 6). During rewarming, MDA formation increased significantly and reached a 1.4-fold higher value compared to control at the end of the rewarming phase. This suggests that lipid peroxidation occurred only during rewarming of the cooled tissue. The rise in MDA formation was minimal in the SOD plus catalase treated group.

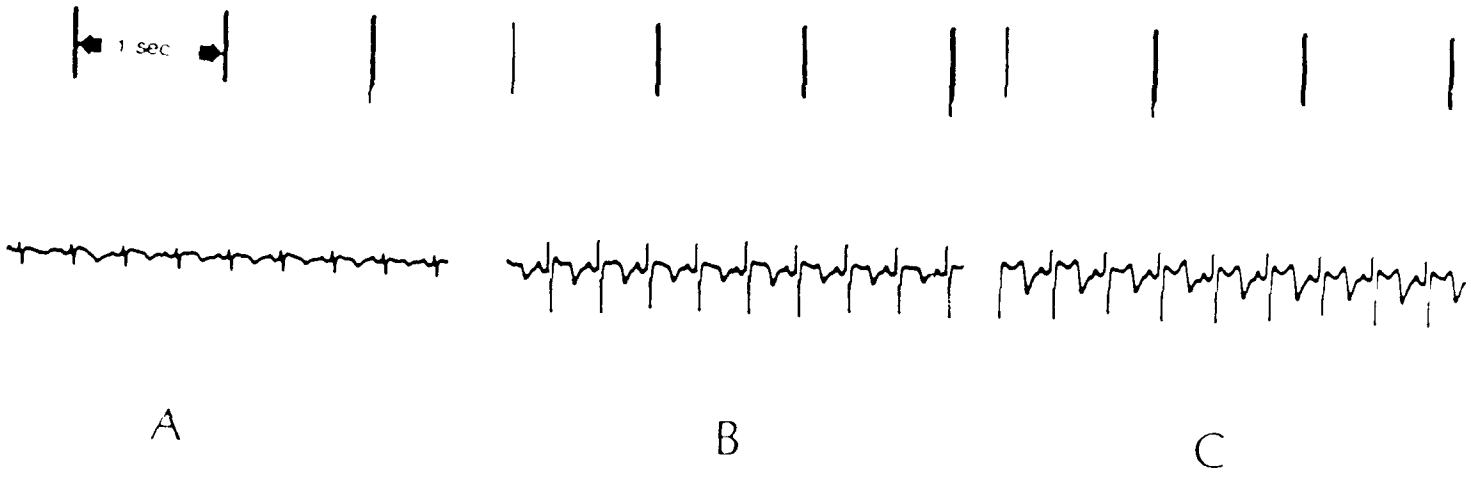
Effects of Rewarming on Membrane Phospholipids

Table 1 shows the effect of rewarming on the contents of membrane phospholipids. There was a reduction in totoal phospholipids during rewarming compared to control, but the difference was not statistically significant. However, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents

were decreased significantly, accompanied by increased LPC in the rewarmed tissue. The decrease in phospholipid content was inhibited by SOD and catalase treatment.

Estimation of Free Radical by HPLC

In some experiments, salicylate was injected through the femoral vein to trap any $OH^{\cdot}$ radical which might be generated in the tissue. We assayed the $OH^{\cdot}$ prior to cooling and at the onset of rewarming, when ice was removed from the leg and blood flow began to rise. We choose this point because oxygen-derived free radicals are known to be produced at the onset of reperfusion of an ischemic organ. The results are shown in Figure 7. The $OH^{\cdot}$ signal increased about 3-fold in the cooled tissue (after ice was removed, when blood flow began to rise, as shown in Fig. 2) compared to baseline control values (Fig. 7B compared to 7A). These signals were reduced significantly in the SOD plus catalase group, suggesting scavenging of $OH^{\cdot}$ radical.



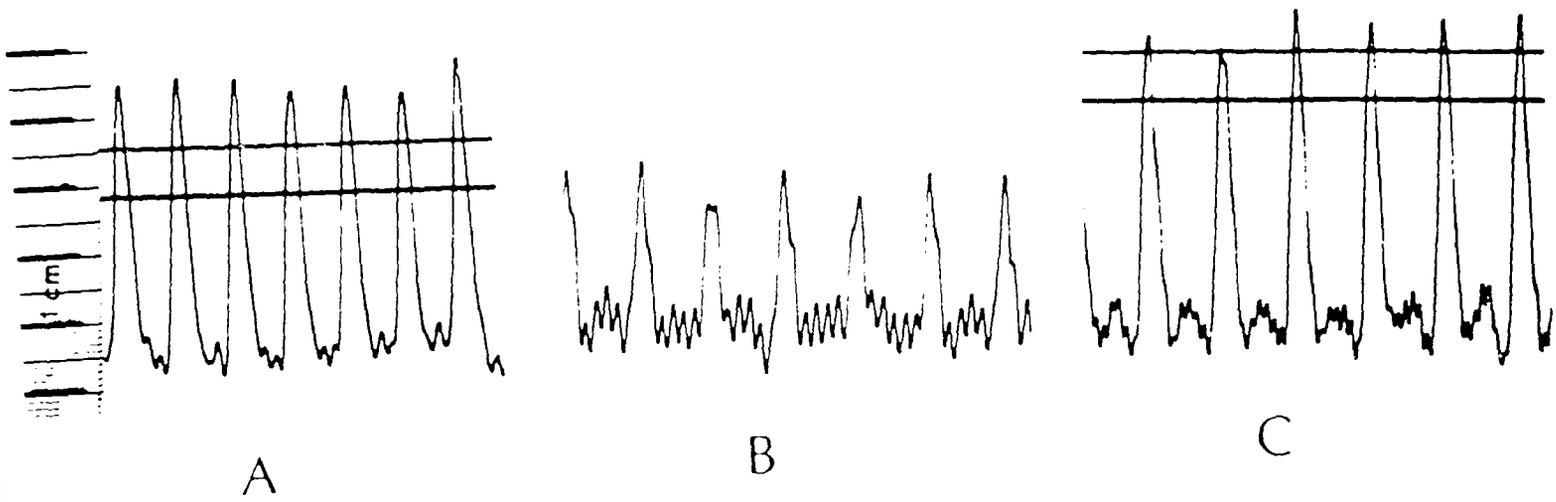
Electrocardiogram of rabbit heart on lead II by connecting the limb leads to simultaneous chart recorder using a chart speed of 25 mm/sec.

(A) Under normal conditions at room temperature.

(B) During cooling of the leg at 0°C.

(C) During rewarming period at 15°C.

FIGURE 1



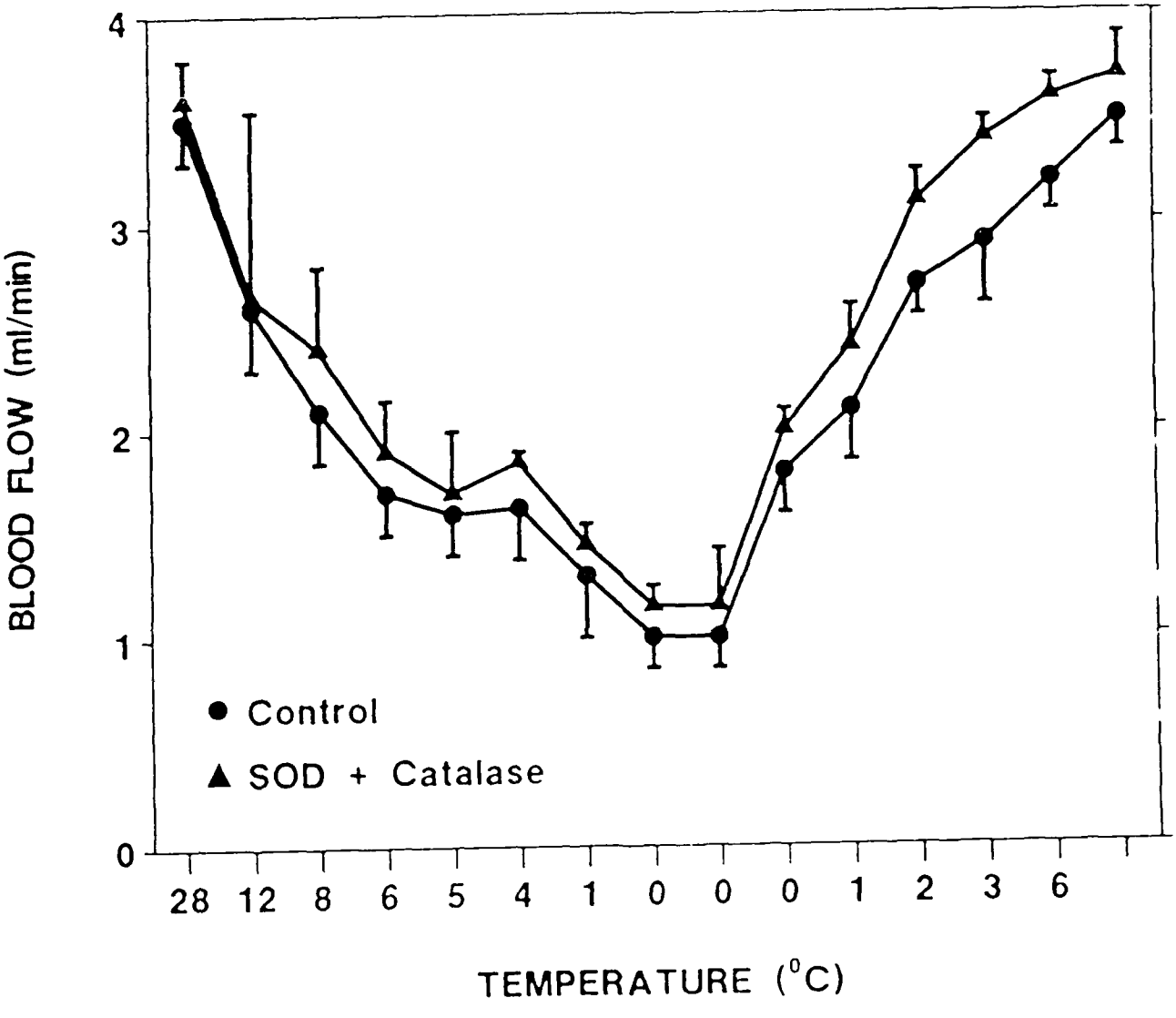
Changes in the pattern of blood flow waves in the femoral artery of rabbit leg using an ultrasonic flow probe.

(A) Under normal conditions at room temperature

(B) During cooling of the leg at 0°C.

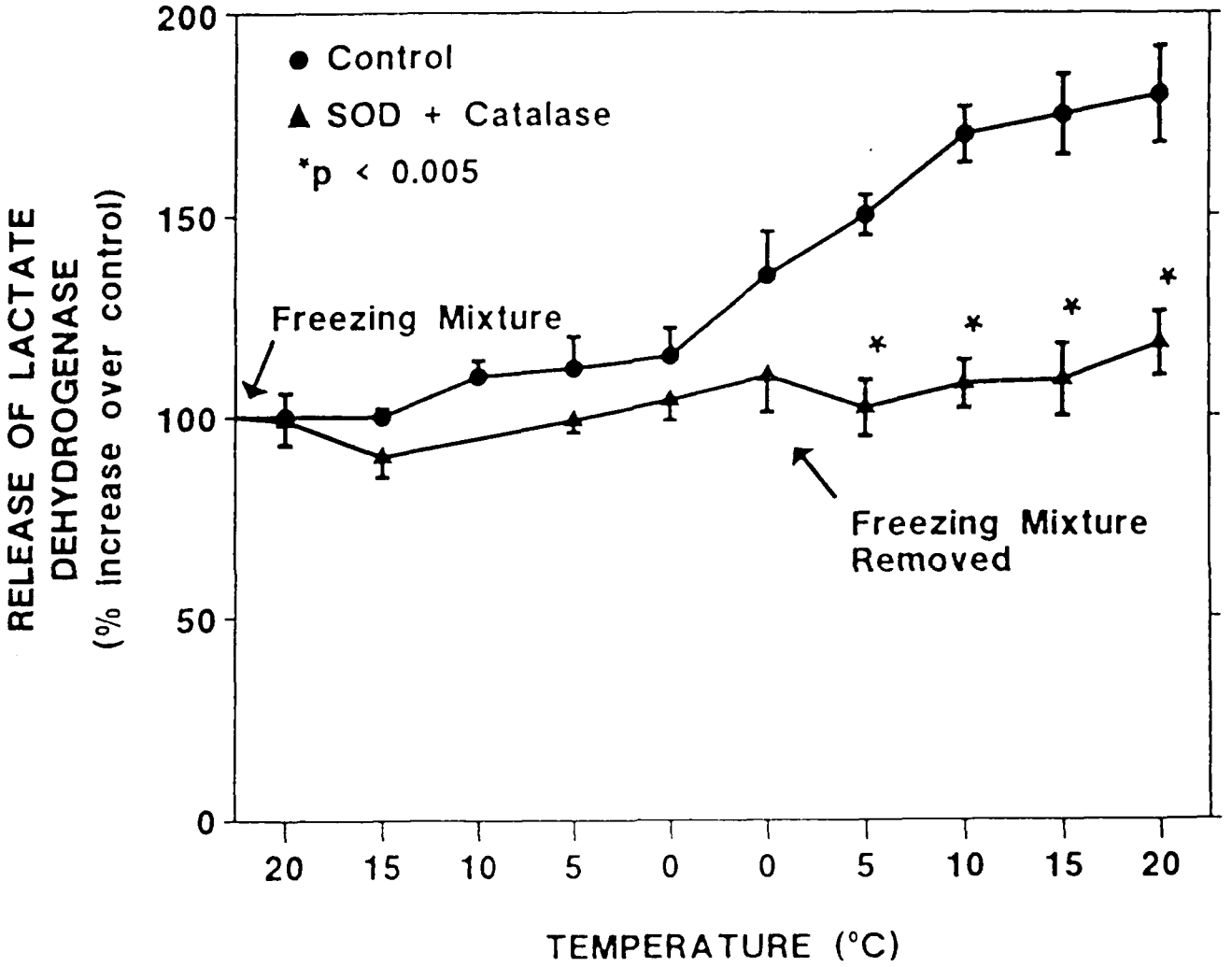
(C) During rewarming period at room temperature.

FIGURE 2



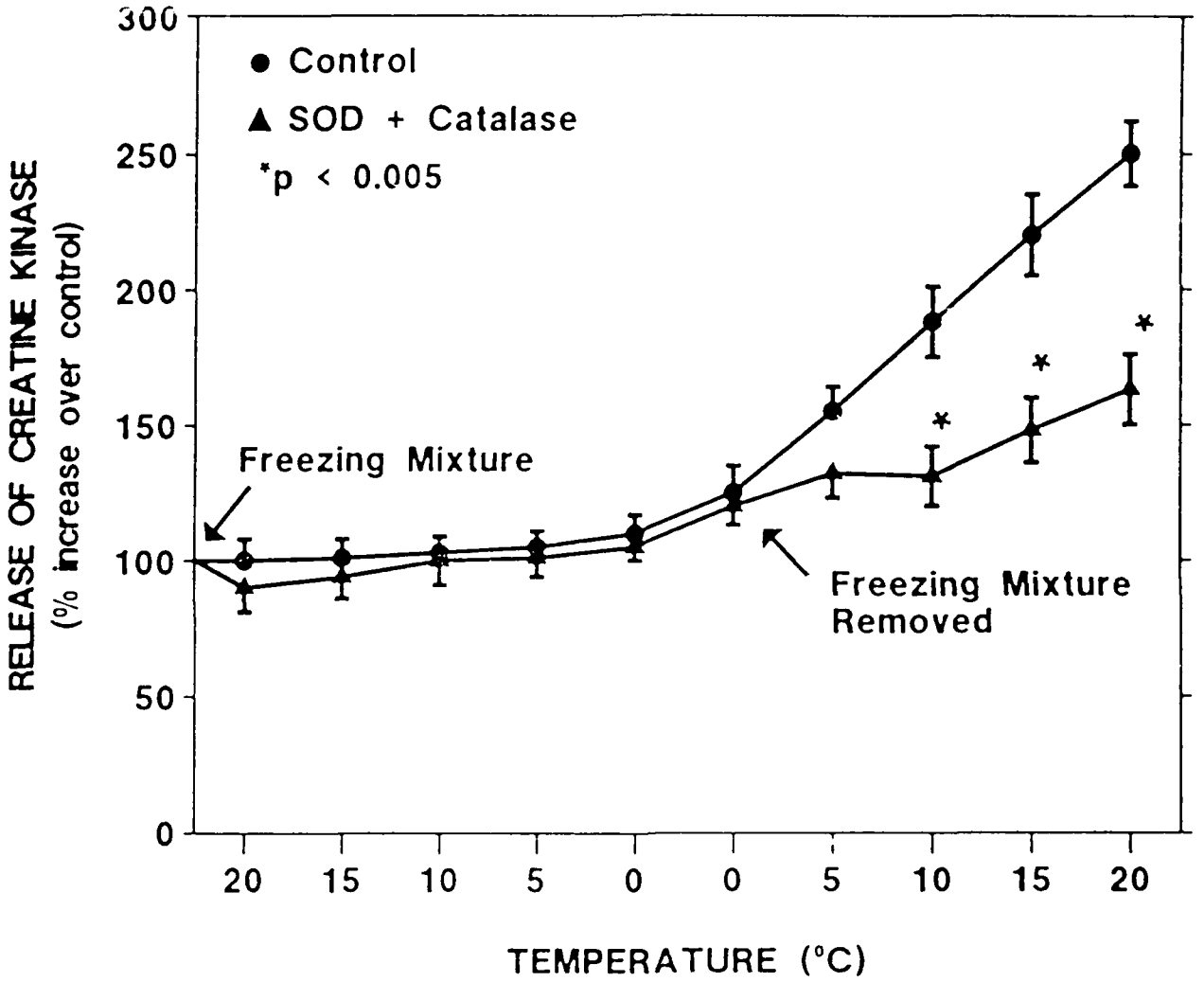
Effects of SOD plus catalase on the changes in the blood flow rate (ml/min) in the femoral artery of rabbit leg during cooling and rewarming. Blood flow was recorded using an ultrasonic flow probe. (0 - 0) control; (● - ●) SOD + catalase.

F I G U R E 3



Effect of SOD plus catalase on the changes in the level of lactate dehydrogenase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming. (0 - 0) control; (● - ●) SOD + catalase.

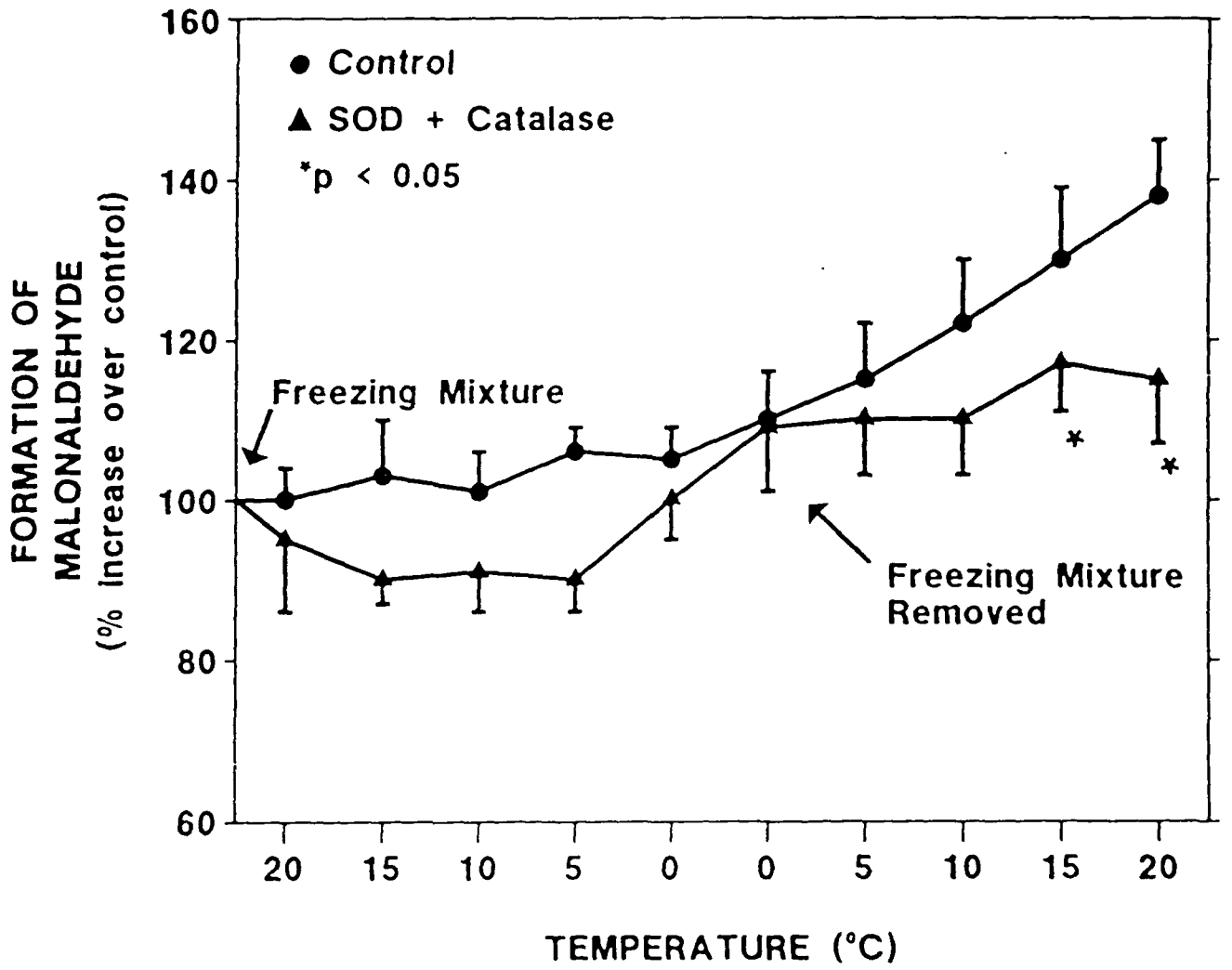
FIGURE 4



Effects of SOD plus catalase on the changes in the level of creatine kinase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.

(0 - 0) control; (● - ●) SOD + catalase.

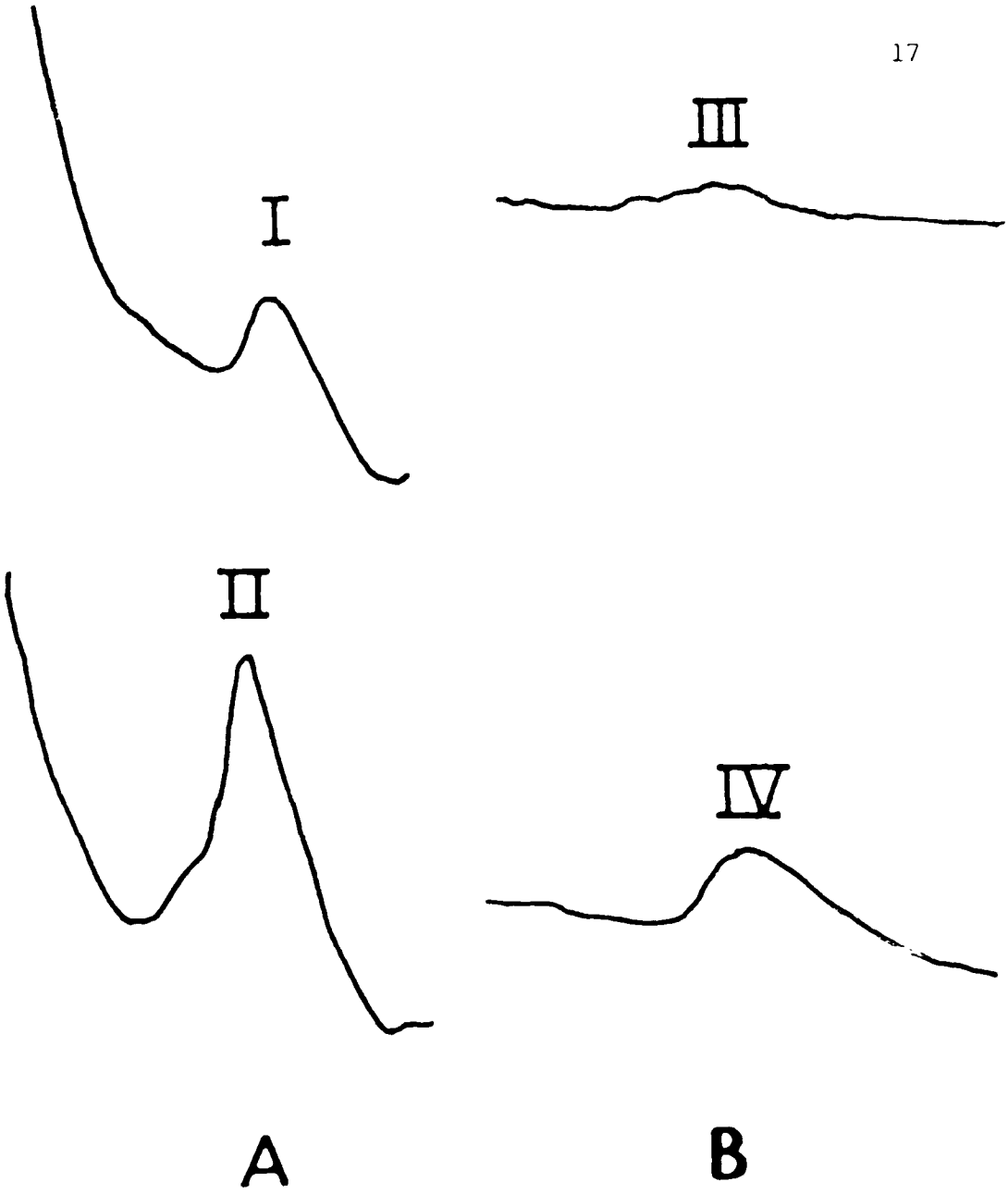
F I G U R E 5



Effects of SOD plus catalase on the changes in the level of malonaldehyde in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming.

(0 - 0) control; (● - ●) SOD + catalase.

FIGURE 6



Chromatograms showing generation of $OH^{\cdot}$ in tissue biopsies obtained from leg. Salicylic acid was injected through the femoral vein, and then biopsies were withdrawn and immediately frozen under liquid nitrogen as described in Methods. Chromatograms were obtained with an HPLC using an electrochemical chemical detector.

I. Control tissue (pre-cooling)

II. Cooled tissue at the onset of rewarming.

III. SOD-treated control (pre-cooling).

IV. SOD-treated cooled tissue at the onset of rewarming.

F I G U R E 7

TABLE 1. EFFECTS OF SOD PLUS CATALASE ON THE BREAKDOWN OF MEMBRANE PHOSPHOLIPIDS IN THE COOLED-REWARMED LEG.

	CONTROL	SOD + CATALASE
Total phospholipids	8.29 ± 0.44	9.8 ± 0.80*
Phosphatidylcholine	4.23 ± 0.48	5.7 ± 0.74*
Phosphatidylethanolamine	1.9 ± 0.34	2.1 ± 0.28
Phosphatidylinositol	0.86 ± 0.13	1.0 ± 0.04
Lysophosphatidylcholine	0.07 ± 0.02	0.01 ± 0.01*

Rabbit leg was subjected to cold exposure and rewarming in the presence or absence of SOD and catalase, as shown in Figure 2. After the experiment, tissue biopsies were withdrawn, lipids extracted, and phospholipids separated and assayed as described in Methods. Results are expressed as Means ± SEM of six experiments in each group.

*$p < 0.05$ compared to control group.

CONCLUSIONS

1. The results of our study demonstrated for the first time that rewarming of cooled tissue is subjected to "reperfusion injury".
2. The presence of oxygen-derived free radicals ($OH^{\cdot}$) has been implicated in cooled-rewarmed tissue.
3. The results also suggest that oxygen-derived free radicals play a significant role in the "rewarming injury".
4. The treatment with free radical scavengers such as SOD and catalase, removed the presence of $OH^{\cdot}$ simultaneously reducing the "rewarming injury".
5. This novel concept of "rewarming injury" and the role of free radicals in such an injury is likely to be an important step in understanding the pathophysiology of NFCI.

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